

Inactivation of an X-linked transgene in murine extraembryonic and adult tissues

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SUMMARY

Transgenes located on the X chromosome have been used to study the mechanisms involved in X-chromosome inactivation. Analysis of the transgenic mouse strain M-TK $neo1$ carrying a neomycin resistance gene inserted in the X chromosome showed that, in adult somatic tissues, this transgene is subject to X-inactivation and to de novo methylation as other endogenous X-linked genes.

During mouse embryogenesis, X-linked genes show a preferential paternal inactivation in extraembryonic tissues, whereas these genes are subject to random inactivation in embryonic tissues. It has been suggested that, in the mouse, the extraembryonic tissues carry a parental imprint at the time of inactivation.

The study of the *neo* transgene expression in extraembryonic endoderm has shown not only that *neo* is inactivated but also that, at the RNA level, paternal inactivation of the transgene seems essentially complete.

The differences between our results and previously obtained results with a mouse α -fetoprotein transgene, which was only inactivated in neonatal tissues but not in extraembryonic tissues, are discussed.

Key words: X-chromosome inactivation, transgene, methylation, extraembryonic endoderm and mesoderm

INTRODUCTION

Dosage compensation in mammals is achieved by inactivation of one of the two X chromosomes in female cells resulting in a single active X chromosome being present in both XX females and XY males (Lyon, 1961). This process takes place during early embryogenesis and seems associated with cell differentiation (Monk and Harper, 1979). X-inactivation in the mouse first occurs in cells of the trophoderm and the primitive endoderm at 3.5 and 4.5 days of gestation, respectively. In these cells that will participate in the formation of the extraembryonic tissues, the X chromosome of paternal origin is preferentially inactivated (Lyon and Rastan, 1984; Takagi and Sasaki, 1975; West et al., 1977). X-inactivation then occurs between 5.5 and 6 days of gestation in the primitive ectoderm (epiblast), which will give rise not only to the three germ layers of the fetus but also to the extraembryonic mesoderm (which is part of the yolk sac). In all these tissues, X-inactivation occurs at random (Gardner and Lyon, 1971; Rastan, 1982; Takagi, 1974). Once it has occurred, X-chromosome inactivation is both highly stable and heritable in a clonal fashion.

This process is thought to be under the control of a region, acting in *cis*, called the X-inactivation center (*Xic*) located by genetic and cytogenetic studies on the X chro-

mosome distal to the T(X:16)16H translocation breakpoint within band X_D (Avner, 1989). The random inactivation observed in adult tissues can be slightly influenced by different alleles of a putative locus called *Xce* (X controlling element), which maps to the same span as the postulated inactivation center itself (Cattanach, 1975). Recently, a human gene, *XIST* (inactive X-specific transcript) and its murine homologue *Xist* have been identified and localised to the *XIC* candidate region in humans and mouse (Borsani et al., 1991; Brockdorff et al., 1991; Brown et al., 1991). *Xist* displays an unusual pattern of transcription in that it is transcribed exclusively from the inactive X chromosome. The location of this sequence to the region of *Xic* and its unique pattern of expression has led to suggest that it may play an important role in the inactivation process, and may possibly correspond to the X-inactivation center itself.

Although the fundamental mechanism of this regulatory process involving *cis* control over long genetic distances is not yet understood, it has been shown that in adult tissues, genes present on the X chromosome are subject to specific methylation, linked to the inactivation process. House-keeping genes such as *Hprt* and *Pgk-1* are hypermethylated in the 5' portion of the gene when present on the inactive X (Hansen and Gartler, 1990; Keith et al., 1986; Lock et al., 1986; Wolf et al., 1984; Yen et al., 1984). This methyl-

ation process seems to occur after the initial steps of chromosome inactivation (Lock et al., 1987) as a secondary mechanism involved in the maintenance of the inactivation in adult tissues.

Additional information concerning the inactivation process has come from the analysis of autosomal material either inserted or translocated into the X chromosome. An apparent spreading of inactivation into autosomal sequences was shown, although this spreading can be limited or incomplete (Lyon, 1983; Russell, 1983).

A number of transgenes located on the X chromosome have been used as models to analyse the inactivation process. The results obtained are not completely coherent: whilst the chicken transferrin transgene studied by Goldman et al. (1987) appears to escape inactivation in adult tissues, both a mouse β -fetoprotein transgene (Krumlauf et al., 1986) and a rat vasopressin transgene (R. Behringer, unpublished results) show correct regulation of expression when present on the inactive X chromosome in adult tissues. One hypothesis advanced to explain the inactivation escape of the chicken transferrin gene was that the non mammalian chicken genome (in which X-inactivation does not occur) either lacks specific signal sequences or is resistant to X-inactivation in the mouse. It was therefore of interest to study the inactivation sensitivity of a non eukaryotic gene which had been integrated into the X chromosome. One such transgene is the bacterial neomycin resistance gene (*neo*) present in the transgenic mouse strain M-TKneo1 (Stewart et al., 1987).

We analysed the regulation of expression of this transgene on the inactive X chromosome in both adult somatic tissues and extraembryonic tissues; our results show that inactivation of the *neo* transgene as well as methylation of its internal CpG sites are similar to those of endogenous X-linked genes. Furthermore, we have shown that, at the level of transcription, paternal inactivation of the transgene seems complete in the extraembryonic endoderm.

The study of this *neo* transgene clearly establishes the capacity of non eukaryotic sequences to respond to the X-chromosome regulation not only in adult somatic cell lineages but also in extraembryonic lineages.

MATERIALS AND METHODS

Mice

The M-TKneo1 transgenic strain obtained after retroviral infection of embryo cells was derived from the ICR strain (Stewart et al., 1987). The retroviral vector MMCV-*neo* (Wagner et al., 1985) is present as a single copy on the X chromosome in this strain (see map in Fig. 3C) and carries the neomycin phosphotransferase gene from Tn5 under the control of an internal herpes simplex virus thymidine kinase (TK) promoter. This vector also contains a *v-myc* gene under the control of the LTR sequences. All M-TKneo1 animals are homozygous for the transgene.

The T(X;16)16H translocation (Searle's translocation) mouse strain, the XO female mice and the C3H/He-*Pgk-I^a* mouse strain were obtained from the breeding facility of the Pasteur Institute.

In mice carrying the Searle's translocation, the reciprocal translocation has occurred between the X chromosome and chromosome 16: in cells from adult females heterozygous for this

translocation, both segments of the rearranged X are active, whereas the normal X chromosome is inactive.

In situ hybridisation

The pSV2neo plasmid was labeled by nick translation to a specific activity of 1.0×10^8 disintegrations/minute/ μ g. Lymphocytes from homozygous M-TKneo1 female mice were cultured under standard conditions in the presence of Concanavalin A for 72 hours at 37°C, 5-bromo-deoxyuridine being added for the final six hours of culture to ensure good post-hybridisation chromosomal R-banding.

Metaphase cells were hybridised as previously described (Dautigny et al., 1986). Slides were covered with Kodak NTB2 nuclear track emulsion and exposed at 4°C. After development the chromosome spreads were first stained with buffered Giemsa solution and cell metaphases photographed. R-banding was then performed using the fluorochrome-photolysis-giemsa (F. P. G.) technique and the metaphases rephotographed prior to analysis.

RNA extraction and northern analysis of adult organs

Total RNA from adult organs was homogenised in guanidinium thiocyanate and purified by centrifugation on a cesium chloride cushion according to described methods (Chirgwin et al., 1979).

For northern analysis, 20 μ g of total RNA were denatured at 68°C for 10 minutes in 50% formamide, 2.2 M formaldehyde, 5 mM sodium acetate, 1 mM EDTA, 20 mM MOPS pH 7.0 and separated by electrophoresis in 1% agarose, 2.2 M formaldehyde gel in MOPS buffer (Sambrook et al., 1989). The RNAs were transferred to a nylon membrane (Hybond N, Amersham) in 10 \times SSC (1 \times SSC is 0.15 M NaCl, 0.015 M sodium citrate) and crosslinked to the dry filter by UV irradiation (0.16 kJ/m²) according to Church and Gilbert (1984).

DNA preparation and Southern analysis

Genomic DNA was isolated from adult organs as described (Sambrook et al., 1989). After restriction enzyme digestion, DNA was separated on 0.8% agarose gels and Southern blotted to nylon membranes (Hybond N, Amersham). DNA was crosslinked to the dry filters by UV irradiation.

Probes and hybridisation

The neomycin probe used for northern and Southern analysis was a 1.1 kb *Bam*HI-*Bgl*III fragment from pSV2neo. The probes were labeled with [³²P]dCTP (Amersham) using the random priming method (Feinberg and Vogelstein, 1984). The β_2 -microglobulin probe is a 300 bp *Eco*RI fragment from a mouse β_2 -microglobulin cDNA clone (Daniel et al., 1983) kindly provided by G. Gachelin (Institut Pasteur, Paris).

Prehybridisation and hybridisation were performed in Church buffer (Church and Gilbert, 1984; 1 mM EDTA, 0.5 M NaHPO₄, pH 7.2, 7% NaDodSO₄) at 65°C overnight. Blots were washed in 2 \times SSC for 15 minutes at room temperature, then 2 \times SSC for 30 minutes at 65°C and 0.4 \times SSC for 30 minutes at 65°C and exposed to Kodak XAR-5 films with an intensifying screen at -70°C.

RNA extraction of yolk sac endoderm and mesoderm

Reciprocal crosses between transgenic M-TKneo1 animals and C3H/He-*Pgk-I^a* mice were established, females tested daily for the presence of vaginal plugs and embryos taken at 14.5 days post coitum. These embryos were sexed under the microscope by analysis of the genital ridges. Individual female yolk sacs were rinsed in PBS and incubated for 2 hours at 4°C in 2.5% pancreatin, 0.5% trypsin, 0.5% polyvinylpyrrolidone, the reaction being stopped by a 10 minute incubation in DMEM medium supplemented with 10% fetal calf serum. Mesoderm and endoderm were then separated using watchmaker's forceps (Hogan et al., 1986). Pools of female mesoderm or endoderm were flash frozen in liquid nitrogen and kept at -80°C.

Total RNA was extracted with acid phenol/chloroform from pools of 5 endoderms or mesoderms homogenised in guanidinium thiocyanate according to described methods (Chomczynski and Sacchi, 1987). After precipitation with isopropanol, samples were treated with RNase-free DNase for 15 minutes at 37°C and reprecipitated after phenol/chloroform extraction. The average yield of total RNA from a single yolk sac varied between 20 and 40 µg. This method of RNA extraction proved to be more efficient with small quantities of material than the guanidinium thiocyanate/cesium chloride method.

RNase A mapping

The *Bam*HI-*Hind*III fragment from the pSV2*neo* plasmid was subcloned into Bluescript KS. This plasmid was linearised with *Nco*I and in vitro transcribed with T7 polymerase (Promega Biotec), using [³²P]CTP (Amersham): the labeled antisense RNA probe is 633 nt long (including 55 nt from the multiple cloning region of Bluescript).

This probe (2×10⁵ disintegrations/minute/sample) was hybridised with 20 µg of either yolk sac endoderm or mesoderm RNA in 20 µl of 75% formamide, 0.5 M NaCl, 1 mM EDTA, 20 mM Tris pH 7.5 overnight at 45°C after a denaturation step of 15 minutes at 85°C. Hybrids were treated with RNase A (Sigma, 40 µg/ml) and RNase T1 (Sigma, 2 µg/ml) in 0.3 M NaCl, 5 mM EDTA, 10 mM Tris pH 7.5 at 37°C for 30 minutes. After proteinase K treatment (0.3 mg/ml in 0.1% NaDodSO₄ at 37°C for 30 minutes), phenol/chloroform extraction and ethanol precipitation, the samples were loaded on 8 M urea 6% acrylamide gels in borate buffer.

mRNA quantification by RT-PCR

10 µg of yolk sac endoderm or mesoderm RNA were reverse transcribed with 10 units of AMV reverse transcriptase and 2 µg of random hexamer primers according to Kawasaki (1990). Samples were treated with DNase-free RNase (10 µg) for 15 minutes at 37°C and ethanol precipitated. For each RNA sample, a control experiment was carried out in parallel omitting the reverse transcriptase. Serial dilutions were prepared from each cDNA sample (500, 50 and 5 ng) and amplified for 20 cycles using Taq polymerase (Cetus) and 0.2 µmol of the primers (92°C, 20 seconds; 65°C, 30 seconds; 70°C, 1 minute) in a total volume of 50 µl.

30 µl of each reaction product were electrophoresed on a 2% agarose gel, transferred to Hybond N⁺ (Amersham) and hybridised to random primed *neo* or actin probes (*Pst*I fragment of pAL41 actin) (Alonso et al., 1986). These PCR conditions (low number of cycles and transfer of the amplified products to a membrane, followed by hybridisation) are such that amplification of cDNAs was linear and dependent on the concentration of the initial RNA.

The *neo* primers used were:

5 GTCCTGATAGCGGTCCGCA3 and
5 GTGTTCCGGCTGTCAGCGCA3 as previously described (Kim and Smithies, 1988).

The actin primers used were:

5 ACCCACACTGTGCCATCTAC3 and
5 CTTCATGGTGCTAGGAGCCAG3 (Alonso et al., 1986).

RESULTS

Localisation of the transgene

In situ hybridisation using the *neo* probe was performed on metaphase spreads of lymphocytes from transgenic M-TK*neo*1 animals. Only metaphases showing silver grains on the X chromosome were analysed. The majority of the grains, i.e. 19 out of a total of 26 (approximately 73%) were detected on bands XD and XE and correspond to the prob-

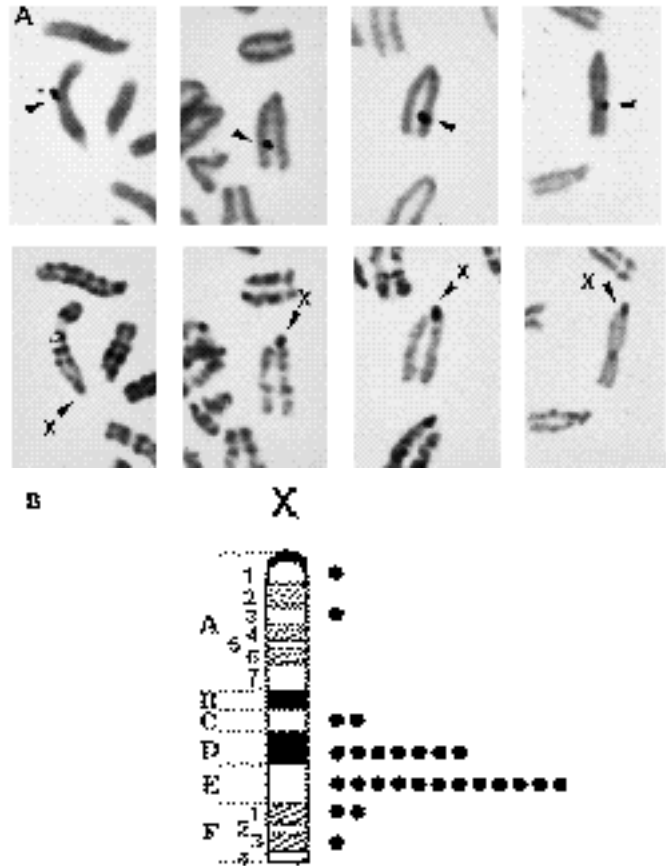


Fig. 1. Localisation of the *neo* transgene on the mouse X chromosome by in situ hybridisation. (A) Individual autoradiographic spreads and corresponding R-banded preparations. (B) Grain distribution on the X chromosome.

able localisation of the transgene on the X chromosome (Fig. 1).

Expression of the neomycin gene in adult tissues

The retroviral vector MMCV-*neo* (Wagner et al., 1985) is present as a single copy in the M-TK*neo*1 strain. Previous studies have shown a tissue-specific expression of the neomycin resistance gene in male and female homozygous transgenic mice (Stewart et al., 1987); a 2.5 kb transcript presumably originating from the internal TK promoter was detected in M-TK*neo*1 heart, lung, muscle, kidney, testis and brain but not in liver, intestine, spleen, thymus and pancreas.

In order to investigate if the *neo* transgene was correctly inactivated in adult tissues, a cross between females heterozygous for the T(X;16)16H translocation (Searle's translocation) and M-TK*neo*1 males was established. Both X,*Xneo* and TX:16,*Xneo* female progeny were obtained. Since the T16H translocation stock carries the Tabby (*Ta*) coat color marker on the non translocated X, the female progeny carrying the translocation can be easily distinguished by their non Tabby coat color.

In these females (TX:16,*Xneo*), both segments of the rearranged X resulting from the reciprocal exchange between the X chromosome and chromosome 16 remain active and the normal *Xneo* chromosome derived from the transgenic stock is uniformly inactivated in all cells.

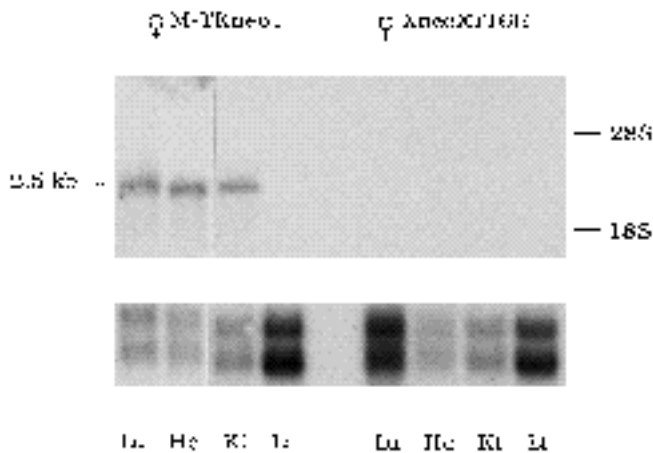


Fig. 2. Expression of *neo* mRNA in female adult tissues. Northern transfer of 20 μ g of total RNA from lung (Lu), heart (He), kidney (Ki) and liver (Li) of a female M-TK*neo*1 mouse and a female X*neo*XT16H mouse was hybridised to a *neo* probe (upper panel) and to a β 2 microglobulin probe as a control (lower panel). The positions of rRNAs are indicated. The *neo* mRNA is 2.5 kb long.

Northern analysis of total RNA from several tissues of these females showed the absence of *neo* expression (Fig. 2, upper panel), whereas the presence of a 2.5 kb transcript was detected in control homozygous M-TK*neo*1 lung, heart and kidney but not liver RNA, as previously described.

These results show that in those adult tissues in which it is expressed, the *neo* transgene is subject to X-inactivation as other endogenous genes.

DNA methylation and X-inactivation in adult tissues

The restriction map of the transgene was established using several enzymes including the methylation-sensitive restriction enzyme *Ava*I. Three *Ava*I sites were localised, one in the *neo* coding region, one in the TK promoter region and one in the *myc* coding region (Fig. 3C).

In order to establish a possible correlation between methylation and the X-inactivation state of the transgene, spleen and brain DNAs from several transgenic animals were digested with *Ava*I and *Hind*III. Southern blot hybridisation of spleen DNAs to the *neo* probe is shown in Fig. 3A.

(1) In both hemizygous M-TK*neo*1 male mice and XO females carrying the *neo* transgene (X*neo*O), a major 1500 bp DNA fragment is detected, due to complete digestion at the *Ava*I sites in the *neo* coding region and TK promoter region. The 2000 bp fragment detected in the spleen DNA of these animals results from partial digestion at the *Ava*I site located in the TK promoter and may be linked to a tissue-specific methylation pattern (see below).

(2) In the TX:16,X*neo* female carrying the *neo* transgene on the normal X chromosome (always inactive in this cross), a single 9000 bp DNA fragment appears (*Hind*III-*Hind*III fragment), indicative of complete methylation of the three *Ava*I sites of the transgene present on the inactive X chromosome.

(3) In the homozygous M-TK*neo*1 female, subject to random X inactivation, the 1500 and 2000 bp fragments

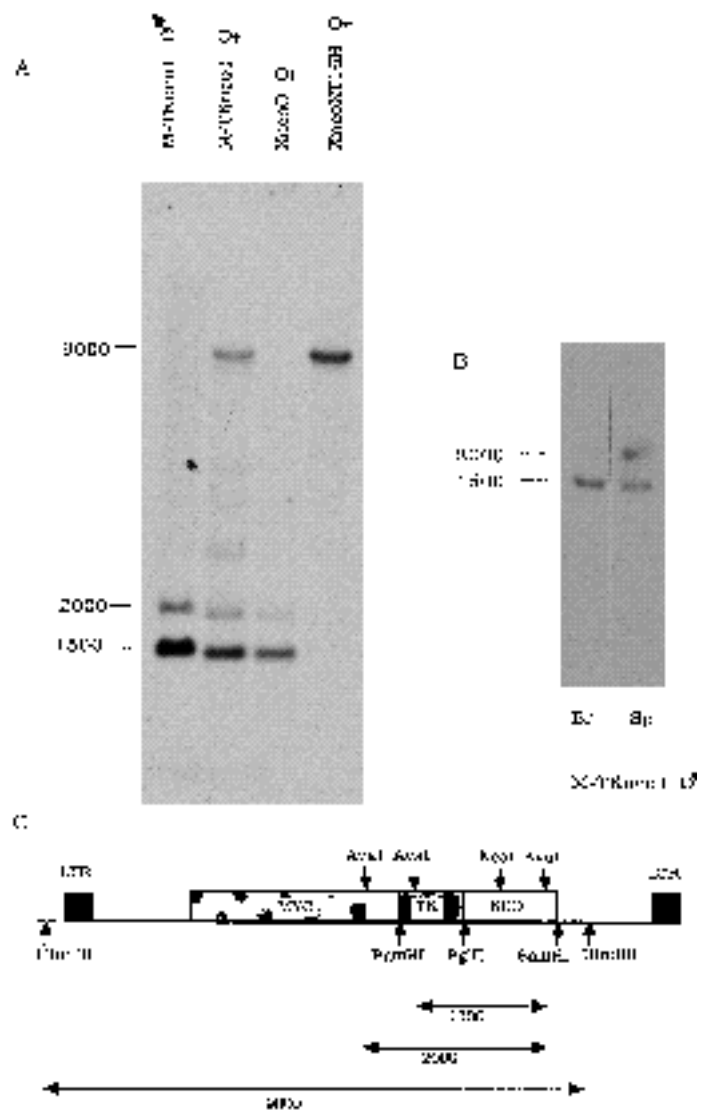


Fig. 3. (A) DNA methylation of the *neo* transgene in adult spleen. Spleen DNAs from homozygous M-TK*neo*1 male and female, from a X*neo*O female and from a X*neo*XT16H female were digested with *Ava*I and *Hind*III and analysed by Southern blot using a *neo* probe. Restriction fragment lengths are shown on the left. (B) Tissue-specific DNA methylation. DNAs from brain (Br) and spleen (Sp) of M-TK*neo*1 male mice were digested with *Ava*I and *Hind*III and analysed by Southern blot using a *neo* probe. (C) Map of the transgene. The MMCV-*neo* retroviral vector carries the *neo* gene under the control of a TK promoter and the *v-myc* gene under the control of LTR sequences. Restriction sites are indicated by short arrows. Long horizontal arrows indicate the length of the restriction fragments obtained after Southern analysis and hybridisation with the *neo* probe.

from the active X chromosome are detected, as well as the 9000 bp fragment from the inactive X chromosome.

Similar results were obtained using two other restriction enzymes, *Sal*I and *Xho*I, which contain a CpG dinucleotide in their recognition site (data not shown).

DNA from spleen (in which the transgene is not expressed) and from brain (in which it is expressed) gave

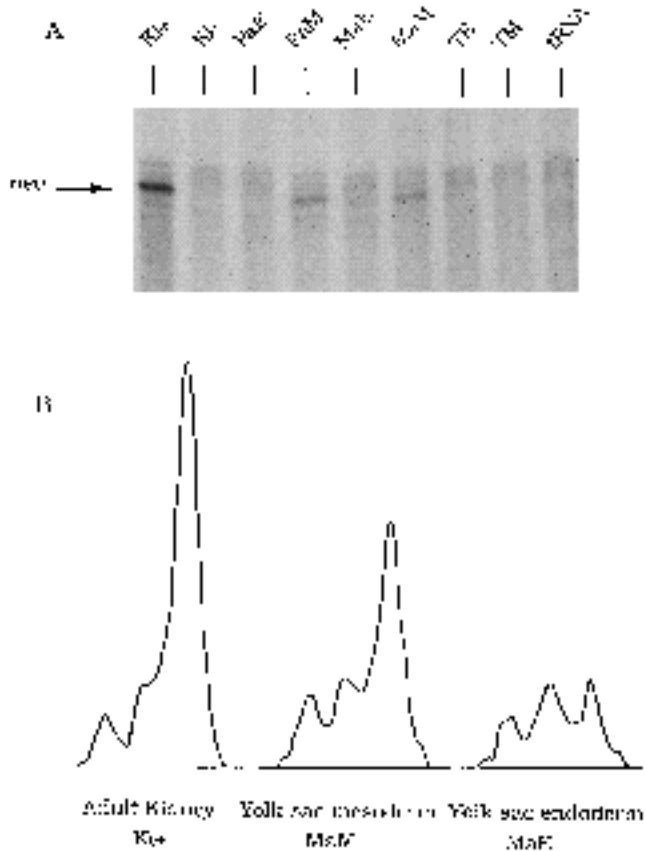


Fig. 4. (A) RNase A mapping analysis of total RNA from yolk sac endoderm and mesoderm. The antisense *NcoI-HindIII neo* RNA probe is 633 nt long of which 578 nt (indicated by an arrow) are complementary to the *neo* mRNA. 20 μ g of RNA from M-TKneo1 adult kidney (Ki+), from a XneoXT16H adult kidney (Ki-), from paternally derived endoderm (PaE) and mesoderm (PaM), from maternally derived endoderm (MaE) and mesoderm (MaM) and from XneoXT16H endoderm (TE) and mesoderm (TM) were hybridised to this probe, digested with RNase A and samples were loaded on 8 M urea 6% acrylamide gel. (B) Densitometric scanning of *neo* mRNA present in adult kidney and in yolk sac mesoderm and endoderm.

identical *AvaI* methylation patterns except for the 2000 bp fragment. Figure 3B shows the Southern blot analysis of M-TKneo1 male DNA from these tissues. The 2000 bp fragment present in the spleen is absent from brain DNA, suggesting that methylation of the *AvaI* site located in the TK promoter could be involved in the observed tissue-specific expression of the transgene.

These results suggest that specific methylation of the transgene is associated with inactivation of the X chromosome and in particular that a bacterial gene such as the neomycin resistance gene can be subject, in the context of the X chromosome, to the same DNA methylation modifications as normal X-linked eukaryotic genes.

Expression of the neomycin transgene in extraembryonic tissues

Extraembryonic tissues display a different pattern of regulation compared to embryonic tissues and show preferen-

tial inactivation of the paternal X chromosome. In order to study the expression of the *neo* transgene in extraembryonic tissues, yolk sacs of 14.5 day female embryos from a cross between male and female M-TKneo1 mice were dissected into endoderm (derived from the primitive endoderm) and mesoderm (derived from the epiblast) and total RNA was prepared from these tissues. Since *neo* mRNA levels in yolk sac tissues were undetectable by northern analysis, we performed the more sensitive RNase A mapping technique using a 633 nt *neo* antisense RNA probe synthesised in vitro by T7 polymerase, of which 578 nt are complementary to the *neo* RNA. *Neo* mRNAs were present in both endoderm and mesoderm of M-TKneo1 animals.

Densitometry analysis (Fig. 4B) of the bands detected on the gel revealed a twofold lower level of RNA in yolk sac mesoderm and a fivefold lower level in yolk sac endoderm compared to the amount in adult tissues, in accordance with the difficulty of detection by northern analysis.

In order to determine if the *neo* transgene was inactivatable in extraembryonic tissues, we set up a cross between a T16H female and an M-TKneo1 male and analysed the endoderm and mesoderm from female 14.5 day embryos. Since both X,Xneo and TX:16,Xneo female embryos are obtained from this cross, it was first necessary to analyse RNA from whole embryos in order to identify the TX:16,Xneo embryos characterised by the absence of *neo* expression and the X,Xneo embryos characterised by its expression. Litter sizes were very small and from the three female embryos obtained, only one embryo was found to be TX:16,Xneo. RNA from the yolk sac endoderm and mesoderm of this embryo was analysed by RNase A mapping: no expression of the *neo* transgene was detected in these tissues (Fig. 4, lane TE and TM) indicating that the transgene was inactivatable in extraembryonic tissues.

In order to determine if the transgene was preferentially inactivated in these tissues when present on the paternal X chromosome, yolk sac endoderm and mesoderm were prepared from embryos obtained from the following crosses, in which the transgene was either paternally or maternally derived:

C3H/He-*Pgk-1^a* \times M-TKneo1/*Pgk-1^b* (cross Pa)
M-TKneo1/*Pgk-1^b* \times C3H/He-*Pgk-1^a* (cross Ma).

As a control for expression in these tissues of an endogenous X-linked gene, *Pgk-1^a/Pgk-1^b* isozyme analysis was performed. No expression of the paternally inherited *Pgk-1^b* isozyme was observed in the endoderm cells of animals from cross Pa, whereas expression of both isozymes was detected in the mesoderm cells with predominant expression of the maternally transmitted gene, as previously described, possibly due to the *Xce^c* allele present in the C3H/He-*Pgk-1^a* strain (West et al., 1977) (data not shown).

Total RNA from these tissues was then analysed by RNase A mapping. The 578 nt long protected *neo* mRNA fragment was present in the yolk sac endoderm of embryos from cross Ma (Fig. 4, lane MaE), but not in the yolk sac endoderm of the embryos from cross Pa (lane PaE). The yolk sac mesoderm of embryos from both crosses was also analysed as a control, since X-inactivation occurs at random in these tissues (lane MaM and PaM) and showed as expected the presence of *neo* mRNA.

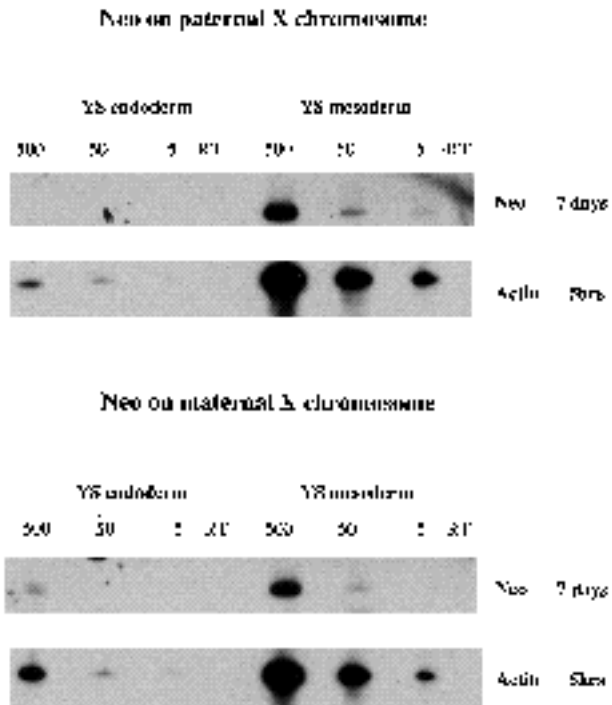


Fig. 5. Quantitative RT-PCR analysis of *neo* expression in total RNA from yolk sac endoderm and mesoderm. Serial dilutions (500, 50 and 5 ng) of reverse transcribed RNA were amplified using *neo* and actin primers. PCR products were separated on 2% agarose gels, transferred and hybridised to *neo* or actin probes. Reciprocal crosses are shown, the *neo* transgene being carried on the paternal X in the top panel and on the maternal X in the bottom panel. Absence of amplification in the -RT samples, where reverse transcriptase was omitted, rules out possible contamination of the samples with genomic DNA.

mRNA quantification by RT-PCR

In order to confirm this result and analyse more precisely the amount of *neo* mRNA present in the yolk sac endoderm and mesoderm, a quantitative RT-PCR experiment was performed on RNA from the Pa and Ma crosses, using *neo* primers and actin primers as a control. No *neo* expression was detected in the YS endoderm RNA when the transgene was carried on the paternal chromosome, whereas *neo* mRNA was present in the endoderm of the Ma cross and in the YS mesoderm of both crosses (Fig. 5).

From these results using RNase mapping and the highly sensitive RT-PCR technique, we conclude that the *neo* transgene is subject to preferential paternal inactivation as other X-linked endogenous genes in the extraembryonic endoderm. Furthermore, the result obtained from the RT-PCR experiment shows for the first time that transcriptional activity of a gene carried by the paternal X chromosome seems apparently totally repressed.

DISCUSSION

Previous studies on transgenes inserted into the X chromosome have shown correct inactivation in adult somatic tissues of the mouse -fetoprotein, SV40 TAG and rat vasopressin genes (Behringer et al., 1988; Krumlauf et al., 1986;

R. Behringer, unpublished results). Only the chicken transferrin gene appears to escape inactivation (Goldman et al., 1987). This observation has been explained in several ways. The gene could have been inserted into a non-inactivated region of the mouse X chromosome. Several human genes such as *MIC2* (Darling et al., 1986), *ZFX* (Schneider-Gädicke et al., 1989), *RPS4* (Fisher et al., 1990) and *AIS9* (Brown and Willard, 1989) have for example been shown to escape inactivation. Although the murine homologs *Zfx*, *Rps4* and *Ais9* are subject to inactivation in mouse (Adler et al., 1991; Ashworth et al., 1991; Zinn et al., 1991), it cannot be excluded that some murine X-linked genes may escape inactivation. The chicken genome (not subject to inactivation) could also lack the correct signals for inactivation in the mouse or could contain repressor sequences inhibiting X-inactivation, therefore suggesting the presence of specific domains or sequences involved in inactivation. The third hypothesis that the inserted unit of 187 kb is too large to be subject to the signals of X-inactivation is unlikely since this is in contradiction to studies on X-autosome translocations and insertions. Autosomal segments much larger than the 187 kb unit of the transferrin gene can, if linked to that part of the X chromosome carrying the inactivation center, be inactivated, probably due to a spreading phenomenon (Lyon, 1983; Russell, 1983).

The transgene present in the mouse strain M-TK*neo1* is a retroviral vector carrying the bacterial neomycin resistance (*neo*) gene under the control of an internal herpes simplex virus thymidine kinase (TK) promoter. It has been previously shown that the *neo* gene in this transgenic strain was efficiently expressed under the control of this promoter in several male and female adult tissues (Stewart et al., 1987).

Our experiments were designed to determine the localisation of the *neo* transgene on the X chromosome, to analyse its expression in different tissues and to study the level of DNA methylation when present on the inactive X chromosome.

The analysis of the transgene insertion by in situ hybridisation provided evidence of its localization in the D or E band of the X chromosome. Preliminary breeding experiments have shown that the *neo* transgene and the *Pgk-1* gene are tightly linked; this result is in agreement with the cytologically defined position since *Pgk-1* lies proximally to the *DXPas2* marker known to locate to the XD/XE band interface. This region of the X chromosome is known to undergo global X-inactivation. The isolation of mouse sequences flanking the transgene will allow us to determine more precisely the site of integration of the vector and define its positional relationship to other endogenous genes.

Expression of the transgene in adult tissues

The use of a cross between the transgenic animal and a T16H female enabled us to study the regulation of expression of the *neo* transgene in adult tissues. In somatic cells from females heterozygous for the translocation, both segments of the rearranged X are active (Disteche et al., 1981; Lyon et al., 1964; Ohno and Lyon, 1965), whereas the normal X chromosome carrying the *neo* transgene is inactive. In these TX:16,X*neo* females, no expression of *neo* is found in tissues such as lung, heart and kidney (in

which the gene is normally expressed in M-TK*neo1* animals) therefore showing that, in adult tissues, the prokaryotic *neo* sequence can be subject to X-inactivation.

It has been suggested that inactivation spreads outward from the inactivation center in a segmental fashion, using relays or 'way stations' on the chromosome to maintain the inactive state in adult somatic tissues (Gartler and Riggs, 1983).

If this is true, then the M-TK*neo1* transgene would have integrated into the X chromosome in a segment that is under the control of these putative relays and would therefore be correctly inactivated at least in adult tissues. This correct regulation also implies that the prokaryotic derived DNA does not contain any sequence capable of inhibiting inactivation.

Transgene DNA methylation

DNA methylation has been suggested as a maintenance mechanism involved in the X-inactivation process. Since the *neo* transgene was correctly inactivated in adult tissues, we investigated the methylation pattern of this exogenous gene and have shown that the *AvaI* restriction sites located within the transgene are methylated when present on the inactive X. This point is particularly interesting since it shows that a bacterial gene, normally only subject to restriction modification methylation, can not only be subject to inactivation like endogenous murine genes on the X chromosome but can also undergo specific methylation of CpGs like other genes on the inactive X.

Analysis of the methylation pattern in brain (in which *neo* is expressed) and spleen DNAs (in which *neo* is not expressed) shows a partial methylation of the *AvaI* site located upstream of *neo* in the TK promoter of the transgene in spleen DNA. This site had previously been shown to be involved in expression of the thymidine kinase gene (Christy and Scangos, 1982). Since this analysis was performed on male DNA, therefore excluding the involvement of methylation due to X-chromosome inactivation, we suggest that this particular methylation may be linked to the lack of expression seen in the spleen of transgenic animals and in some way related to tissue-specific transcriptional control.

Studies on several transgenes have shown the existence of a germline-specific imprinting of the transgenic locus, which is correlated with a differential methylation of the transgene according to its paternal or maternal origin. This genomic imprinting or methylation status of the transgene does not however seem to reflect the methylation status of the chromosomes, in the case of transgenes integrated in autosomal loci. It has been suggested that the transgene itself, by disrupting a chromosomal structure specifies its own methylation pattern (Reik et al., 1990; Sasaki et al., 1991). All transgenes, with the exception of one (Sapienza et al., 1987) show higher methylation of the transgene of maternal origin: it has been suggested that the absence of methylation in sperm is maintained in subsequent somatic divisions in the egg.

It is interesting to note that in the case of the *neo* transgene, the methylation status of the transgene is not linked to the parental origin of the chromosome; in XO females, the transgene is of paternal origin, whereas in the XY males,

the transgene is of maternal origin; in both cases, the transgene is unmethylated. In the TX:16,*Xneo* females, the transgene is of paternal origin and is methylated. It is therefore clear that the methylation pattern seen with the *neo* transgene is specifically linked to X inactivation and not to a parental imprinting.

It can be concluded from our results that in adult tissues a transgene located on the X chromosome can be subject both to inactivation and to the de novo methylation which possibly acts as a maintenance mechanism on segmental domains of the X chromosome after it has been inactivated (Lock et al., 1987).

Expression of the transgene in extraembryonic tissues

In the yolk sac of the 14.5 day embryo, endogenous X-linked genes show a preferential paternal inactivation in extraembryonic endoderm, whereas these genes are subject to random inactivation in extraembryonic mesoderm (originating from the epiblast). It has previously been suggested that in the mouse the extraembryonic tissues carry a parental imprint at the time of inactivation leading to preferential paternal inactivation. The epiblast tissues, in which inactivation occurs later, undergo random inactivation possibly due to the loss of this imprint (Kaslow and Migeon, 1987).

For the analysis of the expression of the *neo* transgene in the yolk sac extraembryonic tissues, we used the RNase A mapping technique and RT-PCR detection, since the levels of *neo* mRNA were too low to be easily detected by northern analysis.

A comparison of the amounts of *neo* mRNA present in adult and extraembryonic tissues has for instance shown a fivefold reduction in the level of mRNA in the yolk sac endoderm compared to adult tissues. This may reflect a tissue-specific transcriptional control of the transgene in adult and extraembryonic tissues and could possibly be linked to the site of integration of the transgene in the X chromosome.

The cross established between a T16H female and the transgenic male enabled us to determine that the *neo* transgene is inactivatable in extraembryonic tissues, since it is not expressed either in yolk sac endoderm or mesoderm of TX:16,*Xneo* embryos.

The transgene is expressed in the mesoderm (of embryonic origin) of 14.5 day embryo yolk sac from both reciprocal crosses Ma and Pa, in which the transgene is maternally or paternally derived, suggesting that in this tissue *neo* is subject to random inactivation. In contrast, in the endoderm of these yolk sacs (of extraembryonic origin), the transgene is only expressed when maternally inherited. The T16H and these results taken together suggest therefore that not only is the *neo* transgene inactivated in extraembryonic tissues as are endogenous X-linked genes but also that it is subject to parental imprinting and shows preferential paternal inactivation in these extraembryonic tissues.

Many authors have previously described this preferential paternal inactivation; in particular, cytogenetic studies (Rastan and Cattanaach, 1983; Takagi and Sasaki, 1975) and quantitation of *Pgk-1* allozymes (Bücher et al., 1985) have been performed on extraembryonic tissues. Our experi-

ments performed on yolk sac endoderm RNA clearly show that not only preferential but total paternal inactivation of the *neo* transgene is observed in this tissue, within, of course, the limit of sensitivity of the RT-PCR technique.

The *neo* transgene, despite its non eukaryotic bacterial origin, is therefore correctly inactivated in extraembryonic and adult tissues and can be both subject to X-inactivation and imprinted in the germ line as other paternally inherited endogenous X-linked genes. This is in contrast with another X-linked transgene, the β -fetoprotein transgene, which is submitted to X-inactivation in neonatal tissues but not in extraembryonic endoderm when present on the paternally derived X chromosome (Krumlauf et al., 1986).

Several explanations can account for the difference in expression of the *neo* and the β -fetoprotein transgenes. (i) Expression in the yolk sac endoderm may be due to the specific mode of regulation of the β -fetoprotein gene: since the endogenous gene is itself very strongly expressed in the yolk sac, it is possible that transcription factors have access to the transgene and override the signals of inactivation in these tissues (Krumlauf et al., 1986).

(ii) Since functional differences exist between the inactive X chromosome in extraembryonic versus adult tissues (Grant and Chapman, 1988), the inactivation mechanism may be affected by the nature of the insert: the β -fetoprotein transgene consists of several tandem copies of the minigene, whereas the *neo* transgene is present as a single copy retroviral insertion.

(iii) Alternatively, the differences of expression of these transgenes may be linked to a failure of imprinting rather than to X-inactivation itself. Since neither the *neo* nor the β -fetoprotein transgene would be expected to contain intrinsic X-chromosome imprinting signals, this suggests that the sites of integration of transgenes may play a critical role in the regulation of their expression in extraembryonic tissues and that target sequences involved in the control of inactivation and imprinting of X-linked genes may be distinct.

The finding that the *neo* transgene can be correctly inactivated in both adult somatic cells and extraembryonic lineages has important implications for the development of test systems for characterising the various genetic elements involved in the X-inactivation process, including candidate sequences for *Xic* itself.

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